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ISOLATION AND PARTIAL CHARACTERIZATION OF PYRIDOXAL 5′-PHOSPHATE HEMOGLOBINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AS A QUALITY-CONTROL METHOD FOR HEMOGLOBIN-BASED BLOOD SUBSTITUTES★

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SUMMARY

Pyridoxal 5′-phosphate hemoglobin (PLP-Hb), prepared from hemoglobin and a four-fold excess of pyridoxal 5′-phosphate by the method of De Venuto and Zegna [J. Surg. Res., 34 (1983) 205], has been chromatographically resolved into six components via a quaternary ammonium monobead support. On an analytical scale, the separations have been found to be rapid (ca. 50 min) and highly reproducible. The results also indicate that the preparation of PLP-Hb yields a reproducible product ratio. The potential of the analytical method for the routine quality control of blood substitutes derived from PLP-Hb is discussed. All five of the PLP derivatives (components II–VI), isolated and purified via a combination of conventional and preparative monobead anion-exchange chromatography, gave single peaks when analyzed by high-performance liquid chromatography. Total phosphate analyses indicated that components II and III each contain two PLPs per Hb, IV and V four and VI six.

INTRODUCTION

Stroma-free hemoglobin (SFH) has two properties which severely limit its usefulness as an acellular oxygen-carrying substitute for red blood cells. When used in vivo, its excessive oxygen affinity results in insufficient lung to tissue oxygen transport [1] while its dissociation into dimers leads to rapid renal clearance and consequently to a short plasma half-life ($t_{\frac{1}{2}}$ 2–4 h) [2]. These and other limitations have been addressed in numerous reviews [3–7]. Briefly, researchers have, in the past, approached the two major limitations separately. The high oxygen affinity of SFH has been reduced to the desired range by covalently binding pyridoxal 5′-phosphate (PLP) to the diphosphoglycerate (DPG) binding site of hemoglobin (Hb) [8,9]. The plasma half-lives have been increased by polymer-

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izing SFH with glutaraldehyde [10] or by binding SFH to dextran [11], serum albumin [12] or inulin [13]. These half-life extending modifications, however, all result in increased oxygen affinity. Recently, a number of researchers have advocated strategies involving a combination of both types of modifications [6,7,14,15]. This approach is exemplified by the preparation and use of glutaraldehyde-polymerized PLP-Hb (poly-PLP-Hb) which has been shown in animal tests to be a highly promising potential hemoglobin-based blood substitute.

PLP-Hb was first prepared by Benesch et al. [8] by reducing the imines formed under anaerobic conditions from PLP and Hb with sodium borohydride. Using classical chromatographic methods, they isolated two major components: $Hb(PLP)_2$ and $Hb(PLP)_4$. Further characterization indicated that in the former the labels are bound exclusively to the β-1-valines and in the latter, to both the β-1-valines and the β-82-lysines [8]. Since these residues are all found in the DPG binding site [9], both derivatives mimic DPG-Hb complexes by exhibiting reduced oxygen affinity [8].

In an attempt to increase the plasma half-life of PLP-Hb, Greenburg et al. [16] modified the preparative procedures of Benesch et al. [8] by increasing the amount of PLP initially present in the reaction mixtures from two to four equivalents [16]. These procedures have been adopted by others and used to prepare PLP-Hb and poly-PLP-Hb on the large scale required for red blood cell substitute applications [17,18]. The effects of these preparative changes on the composition of PLP-Hb have not thus far been reported. In this paper, we describe the high-performance liquid chromatographic (HPLC) resolution of the PLP-Hb reaction mixture. We have isolated and partially characterized six components of the reaction mixture. The potential utility of the HPLC methodology for PLP-Hb-derived blood substitute quality control is also discussed.

EXPERIMENTAL

All buffers and reagents, unless otherwise specified, were obtained in the highest available purity from Sigma (St. Louis, MO, U.S.A.) or BDH Chemicals (Toronto, Canada). Samples were desalted and concentrated with an Amicon stirred ultrafiltration cell equipped with a PM 10 (10 000 MW cut-off) membrane. Water distilled with a Corning AG-11 system was used exclusively. Outdated human blood was supplied by the Canadian Red Cross.

Preparation of PLP-Hb

Stroma-free hemoglobin, isolated from outdated whole blood as previously described [19], was converted to PLP-Hb by the method of De Venuto and Zegna [17]. Methemoglobin levels, as determined with a Corning 2500 CO oximeter, were generally less than 3%. The reaction mixtures were dialyzed against three changes of 50 m*M* Bis–Tris (pH 7.0) concentrated to 20%, filtered through 0.22-μm membranes and then stored in aseptic vials at 4°C.

Analytical HPLC

The analyses were effected with a Pharmacia Mono Q HR 5/5 column using a Pharmacia fast protein liquid chromatographic (FPLC) system. For optimum

TABLE I

BUFFER GRADIENTS USED IN THE PREPARATIVE ANION-EXCHANGE HPLC PURIFICATIONS OF THE COMPONENTS OF PLP-Hb

Component	Buffer B at 0 min (%)	Buffer B at 10 min (%)	Buffer B at 60 min (%)
II and III	0	12	19
IV	0	14	21
V	0	22	30
VI	0	24	32

resolution, the chromatograms were developed with buffer A (20 m*M* Tris–HCl, pH 8.0) and buffer B (buffer A plus 0.5 *M* sodium chloride) using a linear gradient of 5% B to 40% B over 50 min. For routine analysis, the duration of the gradient was reduced to 25 min. The flow-rate was 1.0 ml/min and the pressure 2.0 MPa. Typically, 500 μg of the PLP-Hb reaction mixture or 75 μg of an individual component in 100 μl of buffer A were injected and the separation was monitored at 405 nm (1.0 a.u.f.s.).

Q-Sepharose Fast Flow separation of PLP-Hb

Pharmacia Q-Sepharose Fast Flow (300 ml) was rinsed with buffer A (20 m*M* Tris–HCl, pH 8.0), packed at 4°C into a 50×3.0 cm column, washed with 1.0 *M* sodium chloride (500 ml) and then equilibrated with carbon monoxide-saturated buffer A (2 l). The carbon monoxide-saturated PLP-Hb reaction mixture (2.6 g) in 20 m*M* Tris–HCl, pH 8.3 (90 ml) was loaded onto the column at 2.0 ml/min and then eluted at 4°C at a flow-rate of 5.0 ml/min with a linear gradient prepared from 800-ml aliquots of carbon monoxide-saturated buffers A and B (buffer A plus 0.3 *M* sodium chloride). Fractions were collected every 3.5 min. The absorbance at 550 nm was obtained from every second fraction to yield the chromatogram shown in Fig. 2.

Component isolations

The Q-Sepharose Fast Flow-derived fraction enriched in the desired component, as deduced by Mono Q FPLC analysis, was concentrated to 0.5 ml, diluted to 100 ml with buffer A (20 m*M* Tris–HCl, pH 8.0 for components II, III and IV and 20 m*M* Bis–Tris, pH 6.8 for V and VI), concentrated to 8.0 ml and then loaded at 4°C at a flow-rate of 1.0 ml/min onto a Pharmacia preparative Mono Q HR 16/10 column which had been equilibrated with buffer A (200 ml). The chromatogram was then developed at room temperature with the gradient of buffers A and B (buffer A plus 0.5 *M* sodium chloride for II, III and IV; buffer A plus 0.2 *M* sodium chloride for V and VI) listed in Table I at a flow-rate of 6.0 ml/min. The effluent was monitored at 280 nm. The fractions containing the main component were pooled, desalted as above and then rechromatographed under the same conditions. The fractions indicated in Fig. 4 were pooled, concentrated to 0.5 ml, dissolved in 100 ml of 20 m*M* Tris–HCl, pH 8.0, reconcentrated to 5.0 ml,

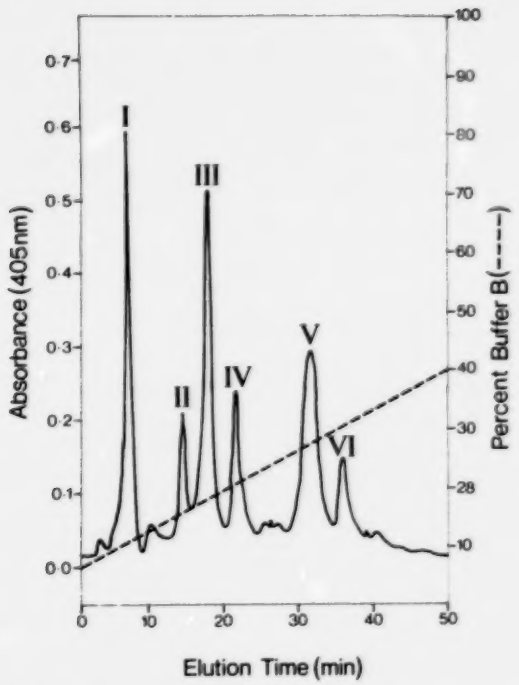

Fig. 1. Analytical anion-exchange HPLC profile obtained from 500 μg of the carbon monoxide-saturated PLP-Hb reaction mixture eluted from the Mono Q HR 5/5 (50×5 mm) column at a flow-rate of 1.0 ml/min. Buffer A is 20 m*M* Tris-HCl (pH 8.0) and buffer B is buffer A plus 0.5 *M* sodium chloride.

saturated with carbon monoxide and then filtered through a 0.22-μm membrane into an aseptic vial. The final concentrations were determined from the absorbance at 540 nm ($\epsilon_{heme} = 14.3\ cm^{-1}\ m^{M-1}$) [20].

Phosphate analyses

The purified specimens were twice analyzed in triplicate for total phosphate using the method of Ames and Dubin [21] with the only modification to the procedure being a four-fold increase in the amount of magnesium nitrate hexahydrate.

RESULTS

Analytical HPLC resolution of the PLP-Hb reaction mixture

Fig. 1 shows a typical analytical chromatogram obtained from the carbon monoxide-saturated PLP-Hb reaction mixture on a Pharmacia Mono Q HR 5/5 column. It clearly shows that, through the use of a simple linear salt gradient and basic elution conditions (pH 8.0), the mixture can be resolved into six components, which we have labelled I–VI. Since Benesch et al. [8] have reported the

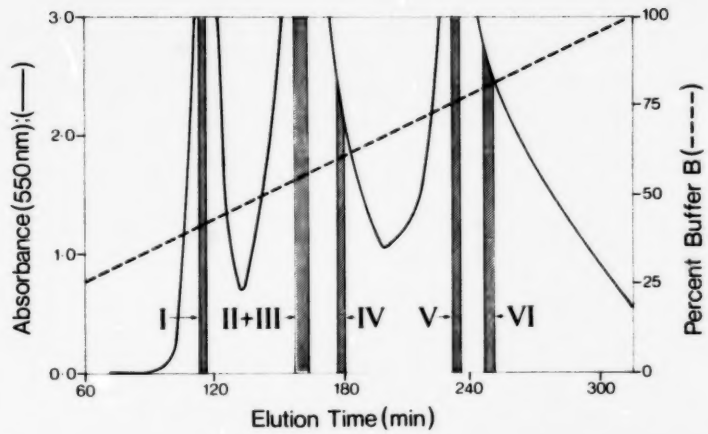

Fig. 2. Preparative chromatogram obtained from 2.6 g of the carbon monoxide-saturated PLP-Hb reaction mixture eluted from a column (50×3 cm) of Q-Sepharose Fast Flow gel at a flow-rate of 5.0 ml/min. Buffer A is 20 m*M* Tris–HCl (pH 8.0) and buffer B is buffer A plus 0.3 *M* sodium chloride. Both buffers were saturated with carbon monoxide. The shaded areas depict the fractions retained for further purification and are labelled according to the component(s) in which they are optimally enriched.

isolation of only three components from their PLP-Hb mixtures, these results suggest that PLP-Hb mixtures prepared by the method of De Venuto and Zegna [17] are more complex.

Isolation of the PLP-Hb components by conventional chromatography

The first step in the component isolation sequence involved a partial resolution of 2.6 g of the PLP-Hb reaction mixture on a column of Pharmacia Q-Sepharose Fast Flow gel. The chromatogram is shown in Fig. 2. Using the analytical HPLC procedure as a guide (see Fig. 1), fractions optimally enriched in each of the components were selected for further purification. These are depicted as shaded areas in Fig. 2 and are labelled according to the enriched component. The HPLC analysis of fraction I gave a single peak which appeared in the chromatogram at the same position as did the major component of SFH (assumed to be HbA). Further purification was, therefore, not implemented.

Isolation of the PLP derivatives of Hb by preparative HPLC

The selected fractions (see Fig. 2) were further purified chromatographically on a preparative Pharmacia Mono Q HR 16/10 column using a shallow salt gradient centered at the salt concentration at which the desired component was found to elute. Fig. 3 shows a typical chromatogram that is obtained from fraction II plus III. The major component was collected, desalted, concentrated and then, to ensure the purity of the specimen, rechromatographed under the same conditions. This set of chromatograms is shown in Fig. 4. Generally, 25 mg of the purified component were so obtained from 100 mg of the corresponding Q-

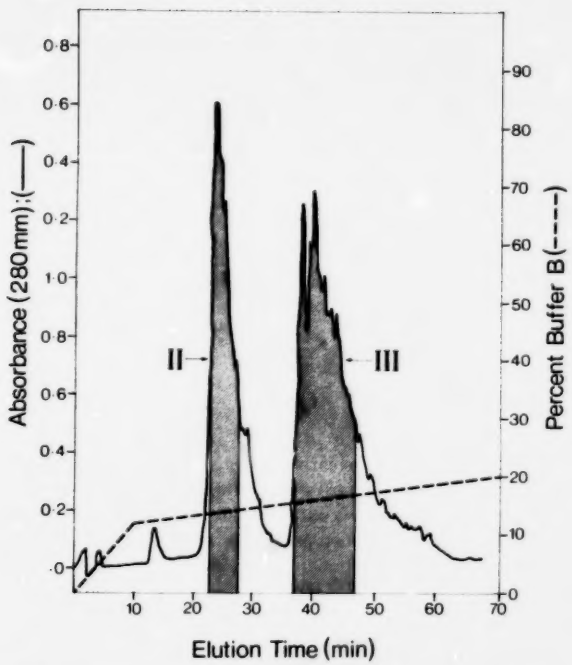

Fig. 3. Preparative anion-exchange HPLC profile obtained from 80 mg of the carbon monoxide-saturated Q-Sepharose derived fraction enriched in components II and III (see Fig. 2) eluted from a Mono Q HR 16/10 column (10×1.6 cm) at a flow-rate of 6.0 ml/min. Buffer A is 20 m*M* Tris–HCl and buffer B is 0.5 *M* sodium chloride in buffer A. The shaded areas depict the fractions of II and III retained for further purification.

Sepharose-derived fraction. When subjected to HPLC analysis, all of the purified components gave single peaks.

Co-chromatography of the isolated components and the reaction mixture

In order to confirm the identities of the isolated components, each was analyzed as an additive to the reaction mixture. The resulting set of co-chromatograms clearly indicated that the isolated components do in fact correspond to the distinct PLP-Hb components identified in the analytical chromatogram. As an example, the co-chromatogram of fraction III and the reaction mixture is shown in Fig. 5.

Partial characterization of the PLP-Hb components

The purified PLP-Hb fractions (I–VI) all gave UV–VIS spectra indistinguishable from carbon monoxy Hb. Specimens of each were analyzed spectrophotometrically for total heme and then chemically for total phosphate. The results, expressed as mol phosphate per mol Hb, are listed in Table II and clearly indicate that fraction I is unmodified Hb, II and III are $Hb(PLP)_2$ species, IV and V are $Hb(PLP)_4$'s and VI is an $Hb(PLP)_6$. Since the addition of PLP labels to Hb

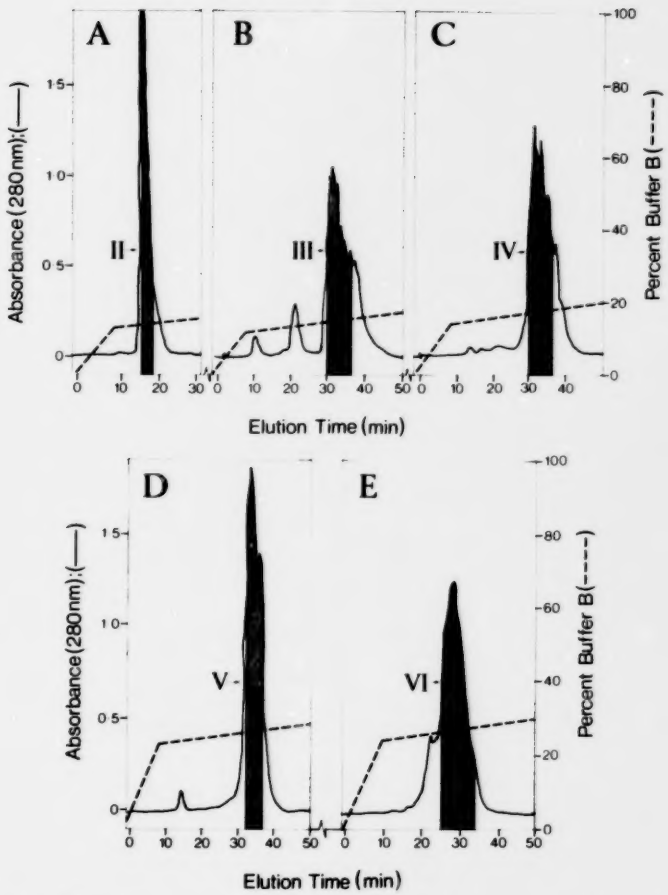

Fig. 4. Preparative anion-exchange HPLC profiles obtained from approximately 40-mg samples of the carbon monoxide-saturated purified components of PLP-Hb eluted from a Mono Q HR 16/10 column at a flow-rate of 6.0 ml/min. For A, B and C, buffer A is 20 m*M* Tris–HCl (pH 8.0) and buffer B is 0.5 *M* sodium chloride in buffer A. For D and E, buffer A is 20 m*M* Bis–Tris (pH 6.8) and buffer B is 0.2 *M* sodium chloride in buffer A. The buffer gradients are listed in Table I. The retained fractions are shown as shaded areas and are labelled according to their contents.

results in an increase in net negative charge, the expected inverse trend between the elution times in the anion-exchange chromatograms and the labelling stoichiometry is indeed observed.

DISCUSSION

The development of monobead HPLC supports has provided protein chemists with high-resolution chromatographic methodology for the separation of complex protein mixtures [22]. Generally, these separations are fast, highly repro-

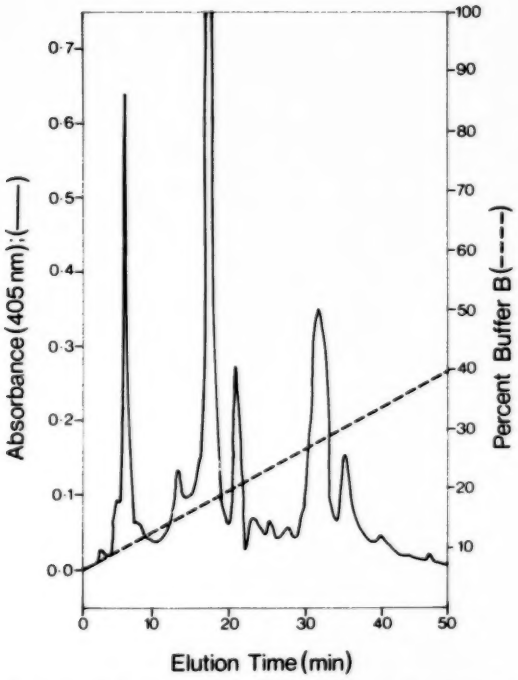

Fig. 5. Analytical anion-exchange HPLC co-chromatogram obtained from the reaction mixture (500 μg) mixed with component III (50 μg) eluted under conditions identical to those used in Fig. 1.

ducible and, consequently, well suited to routine analytical application. Moreover, the ease with which the separations can be effected on a semi-preparative scale facilitates the isolation and subsequent characterization of components of these mixtures. These advantages have been realized by Robinet et al. [23] in their detection and separation of human hemoglobin variants under acidic conditions using a monobead cation exchanger, the Pharmacia Mono S. Comparable separations have been obtained by Moo-Penn and Jue [24] with a Mono Q anion exchanger under basic conditions. We have previously reported the resolution of

TABLE II

PHOSPHATE ANALYSES OF THE COMPONENTS OF PLP-Hb

Component	Moles phosphate/moles Hb
I	0.1 ($\pm$0.2 S.D.)
II	2.1
III	1.9
IV	4.1
V	3.9
VI	6.1
(HbA)	0.0

ATP-modified hemoglobin mixtures with an analytical Mono S column [25] and the separation of glyoxalated and unmodified hemoglobins via a Polyanion SI monobead support [26]. Collectively, these applications demonstrate the utility of monobead supports in the chromatographic separations of hemoglobin mixtures.

To date, only two chromatographic methods for the separation of PLP-Hb mixtures have been reported. Benesch et al. [8] have used conventional means (phospho-cellulose) while Přistoupil et al. [27] have chromatofocused the mixtures of PLP-Hb. The former method requires lengthy development times (24 h) and provides only low resolution. The latter suffers from poor resolution and from complications arising from sample decomposition, as well as the in situ autoxidative formation of the corresponding methemoglobin derivatives. These difficulties, in conjunction with the demonstrated potential of monobead supports for modified hemoglobin separations, prompted us to investigate the chromatographic resolution of PLP-Hb under basic conditions using Mono Q anion-exchange monobeads.

The results of our analytical separations clearly demonstrate that PLP-Hb prepared by the methods used by De Venuto and Zegna [17] and Noud et al. [18] consists of six components. We have found, with one exception which will be discussed below, that different chromatograms obtained from the same PLP-Hb mixture are indistinguishable. As well, we have observed no significant batchwise variation in the relative intensities of the peaks and, therefore, conclude that these authors' preparative methods yield highly reproducible product ratios. Also noteworthy is that indistinguishable chromatograms are obtained from both the oxy and carbon monoxy derivatives of the PLP-Hb reaction products. These features coupled with the high-resolution, rapid chromatographic development and high sensitivity inherent in the analytical method clearly demonstrate the utility of monobead HPLC for the routine analysis of PLP-Hb mixtures prior to their subsequent modification into poly-PLP-Hb.

The reproducibility exception mentioned above is that HbA peak (fraction I) is occasionally observed as a pair of peaks. The new peak is observed at the position of the small shoulder at the beginning of the first peak in Fig. 1 and is formed at the expense of component I. Robinet et al. [23] have described a similar complication in their cation-exchange HPLC (Mono S) analysis of HbA. Under their acidic elution conditions, the appearance of the second peak can be eliminated by simply saturating the sample with carbon monoxide prior to its elution. This remedy fails under the basic conditions used in our analyses and, accordingly, the nature and properties of the phenomenon are currently being investigated.

In order to eliminate difficulties associated with autoxidation, all preparative chromatographic operations directed at the isolation and purification of the individual components were performed on the carbon monoxy derivatives of the reaction mixture. Preliminary results indicated that resolution comparable to that shown in Fig. 1 could be obtained from 150 mg of the reaction mixture on an HR 16/10 Mono Q column. On the desired scale, multiple runs would, therefore, be required. Better resolution, however, could be obtained if a more shallow gradient was used. Since this modification would require much longer elution times and,

as a consequence of the maximum load, still require multiple runs, it was decided to first carry out a large-scale partial resolution of the mixture on a conventional gel (Q-Sepharose Fast Flow). Noteworthy is that the separation requires an elution time of only 6 h. Fractions optimally enriched in each of the components were selected via HPLC analysis and then purified by elution through a Mono Q HR 16/10 column using a shallow salt gradient. A second chromatographic run then provided the isolated components at a high level of purity as deduced from the single peaks obtained from these specimens on HPLC analysis. This preparative method represents a simple, fast and efficient means with which pure components may be isolated from the mixture in sufficient quantities as to allow for their subsequent analysis and characterization. Furthermore, this approach should be generally applicable to the preparative separations of the components of other modified hemoglobin mixtures.

The phosphate analyses clearly indicate that I is unmodified HbA, II and III are $Hb(PLP)_2$ species, IV and V are $Hb(PLP)_4$'s and VI is an $Hb(PLP)_6$. Since III and V are the major modified products, they, in all likelihood, correspond to the doubly and quadruply labelled products isolated by Benesch et al. [8]. The structures of the remaining components (II, IV and VI) are currently being elucidated.

CONCLUSIONS

We have resolved PLP-Hb mixtures via anion-exchange HPLC on a quaternary ammonium monobead support. On an analytical scale, the method has been shown to be simple, fast, highly reproducible, and hence, ideally suited for the routine high-resolution quality-control analysis of PLP-Hb mixtures prepared for blood substitute application. On a semi-preparative scale six pure components have been isolated from the mixtures.

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